In-Vitro Assessment of Antimicrobial and Cytotoxic Activities of *Wulfenia Amherstiana* against the Human Breast Adenocarcinoma (MCF-7) and Human Hepatocellular Liver Carcinoma (Hepg-2) Cell Lines

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Abstract

To investigate the cytotoxic activity against human cancer cell lines and antimicrobial (antibacterial and antifungal) potential of *Wulfenia amherstiana* or *Wulfeniopsis amherstiana* by preparing its ethanolic extract and n-hexane, chloroform, n-butanol and ethyl acetate fractions. Antimicrobial activities were determined through agar well diffusion method. MTT assay was used to assess the cytotoxic activity against human breast adenocarcinoma (MCF-7) and human hepatocellular carcinoma (HepG-2) cell lines. Antibacterial assays revealed that chloroform fraction showed maximum efficacy against gram positive bacteria *Staphylococcus aureus* NCTC 6571 (95.2% inhibition) and against *Pseudomonas aeruginosa* ATCC 10145 (96.2% inhibition). Both Chloroform (90% Inhibition) and n-hexane (80 %Inhibition) fractions were found most efficacious against *Aspergillus flavus* ATCC 32611. The MTT assay revealed considerable cytotoxicities of plant ethanolic extract (IC_{50} = 50 µg/ml, 100 µg/ml against MCF-7 and HepG-2 cell lines, respectively), and chloroform (IC_{50} = 100 µg/ml and 150 µg/ml against MCF-7 and HepG-2 cell lines) and ethyl acetate fractions (IC_{50} = 150 µg/ml and 200 µg/ml against MCF-7 and HepG-2 cell lines). The n-butanol and n-hexane fractions were comparatively less cytotoxic.The present study concluded that the *W. amherstiana* possesses significant antibacterial, antifungal, and cytotoxic activities. The ethanolic extract, its chloroform and ethyl acetate fractions were found moderately cytotoxic proving the plant's anticancer activity.

Key words: Wulfenia amherstiana, Antibacterial, Antifungal, Cytotoxic, Phytochemical screening

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Introduction

All through the ages, people have been dependent on nature for their fundamental needs such as food, source of protection, dressing, transportation, manures, flavors, scents, and drugs [1]. Plants have shaped the foundation of complex customary medication frameworks that have been utilized for several years and keep on providing mankind with new cures. The primary records, composed on mud tablets in cuneiform, are from Mesopotamia and date from around 2600 BC [2] revealed that at that time, oils of various plants such as *Cedrus* species and *Cupressus sempervirens (cypress), Glycyrrhiza glabra* (licorice), *Commiphora* species (myrrh) and *Papaver somniferum* were used for various diseases. These are also used in the present age for treating flu, cough, various infections and inflammation. There are several plants fimilies having biological activities are still to be explored. One such family is *Scrophulariaceae*. This is a family of flowering plants, commonly known as the figwort family which is distributed all around the world, consists of approximately 275 genera and around 5,000 species [3]. *Scrophulariaceae* is a well-known family found in Europe, North America, temperate Asia, and is famous because of its therapeutic properties. A number of medicinal properties including anti- inflammatory, anticancer, antioxidant, antimicrobial, antihepatotoxic, antiviral, anti- hyperlipidemic activity have been credited to this family [4].Many species of this family have been found in Pakistan. One of the species of this family is *Wulfenia amherstiana* (*W. amherstiana*) or *Wulfeniopsis amherstiana*[5], which is still to be explored as no data is available regarding its biological activities. Its only known medicinal use is the healing of muscular pain and relief of fever [6]. Though other species of the family (*Scrophulariaceae*) have been reported for their various biological activities such as antimicrobial and cytotoxic activities. The specie which has been found to exhibit significant antimicrobial activity against both gram positive, gram negative bacterial and fungal pathogens is *limnophilla indica*[7].While the genus *Mazus goodenifolius* has been found to show strong antibacterial activity and minor cytotoxic activity[8]. Another plant knowns as *Scoparia dulcis* has demonstrated significant antifungal, cytotoxic activities and moderate antibacterial potential [9]. These reported antimicrobial and cytotoxic activities of several species of family *Scrophulariaceae* have formed a strong foundation to for our study to evaluate the antimicrobial and cytotoxic potential of *W.amherstiana*.

Materials And Methods

Materials

Bacteria selected for study were *Staphylococcus aureus* (*S.aureus* NCTC 6571), *Staphylococcus aureus* (*S.aureus* NCTC 6571) *Streptococcus pneumoniae* (*S.pneumoniae* NCTC 7466), *Bacillus subtilis* (*B.subtilis* NCTC 8236), *Shigella flexneri* (*S.flexneri* ATCC 12022),*Pseudomonas aeruginosa* (*P.aeruginosa* ATCC 10145), *Salmonella typhi* (*S.typhi* ATCC 6539), fungi under test were *Trichophyton longifusis* (*T.longifusis* Clinical Isolate), *Candida albicans* (*C.albicans* ATCC 2091), *Aspergillus flavus* (*A.flavus* ATCC 32611), *Microsporum canis* (*M.canis* ATCC 11622), *Fusarium solani* (*F.solani* ATCC 11712), *Candidia glabrata* (*C.glabrata* ATCC 60406) and brine

shrimp (*Artemia salina*). All these strains and brine shrimps were provided by PakistanCouncil of Scientific & Industrial Research (PCSIR) laboratories, Peshawar, KPK, Pakistan.Nutrient broth (NB), Nutrient agar (NA), Sabouraud dextrose agar (SDA), Dimethyl sulfoxide (DMSO), Standard antibiotics Cefazolin, Kanamycin and standard antifungal drugs such as amphotericin-B and Miconazole, anticancer drug i.e. Tamoxifen, Methyl Thiazol Tetrazolium (MTT) were purchased from Sigma Aldrich, UK. Various solvents such as Chloroform, Ethyl acetate, n-Butanol, n-Hexane used were made by Merck. Micro plate-well spectrophotometer (Epoch 2 Microplate Spectrophotometer)

Cell lines
In the present study the cell lines used were human breast adenocarcinoma (MCF-7), human hepatocellular liver carcinoma (HepG-2). The cell lines were provided by Pakistan Council of Scientific & Industrial Research (PCSIR) laboratories, Peshawar, KPK, Pakistan.

Methodology
Plant Collection and Identification
W. amherstiana, plant was collected from the hilly areas of Baragali during summer season in August. A taxonomist Dr.Muhammad Ibrar recognized the plant and a voucher specimen (ID No.WA450987) was deposited at the herbarium of Department of Botany, University of Peshawar, Pakistan.

Preparation of Plant extract
The whole plant (weighing 15 kg) was shade dried in fresh air for several days and then chopped into small pieces. The small pieces were pulverized to fine powder and soaked in 4 liters ethanol for 1.5 weeks with time to time shaking. Repeated extraction and concentration of the mixture through rotary evaporator offered 175g of the gummy residue. Furthermore, prepared ethanolic extract was subjected to fractionation or partitioned using different organic solvent like n-Hexane, Chloroform, ethyl acetate and n-Butanol.

Preliminary Phytochemical screening
The crude ethanolic extract of the whole plant was tested for the presence of various biological compounds by various specific chemical tests such as steroids & terpenoids (Liebermann-Burchard test)[10], alkaloids (Mayer's Test), tannins, flavonoids (Aluminum Chloride Calorimetric Method), saponin and glycosides through Keller-Kiliani Test[11].

Antibacterial Assays (Agar well Diffusion Method)
The agar well diffusion test was adopted for testing the bacterial sensitivity against crude extract and its various fractions of *Wulfenia amherstiana*[12]. The test samples (crude extract and its various fractions) in various concentrations 100, 200, 300, 400,500 and 600µg/ml were

dissolved in appropriate solvent i.e. 0.5% DMSO and was poured using a micropipette into appropriately labelled wells made with sterile borer on the NA plates. The plates were left at room temperaturefor 2 hours to allow diffusion of the sample and incubated face upwards at 37°C for 24hr. The zones of inhibition were measured and recorded in triplicates. For positive control *Cefazolin* was used for gram positive bacteria and *Kanamycin* for gram negative bacteria and the solvent was used as negative control.

Antifungal Assay

Antifungal assay was also performed by using Agar well dilution method using SDA plates for all the test fungi and molds. The test samples were dissolved in their respective solvent (0.5% DMSO) to serve as stock solution. Each plate was inoculated with culture of fungi and the required number of well were made with sterilized borer. The test samples (various crude extracts in various concentrations 100, 200, 300, 400,500 and 600µg/ml) poured in to the wells. Each plate is inoculated at optimal temperature 28-30°C and Humidity (40% to 50%) for growth for 7-10 days. The plates were examined for any visible growth of the fungus and the results were recorded in triplicates [12, 13]. The standard antifungal drugs used were amphotericin B and Miconazole as positive control while the solvent 0.5% DMSO was used as negative control.

Cytotoxicity Assay by MTT method

The cytotoxic activities of plant extract and its fractions was assessed by their effects on human breast adenocarcinoma (MCF-7), human hepatocellular liver carcinoma (HepG-2) cells grwoth. The 96-well micro-plate was seeded with MCF-7 8 $\times 10^3$ cells and HepG 1 x 10^4 cells through MTT assay [14].These cells were subjected to incubation at 37°C in the CO_2 incubator for 24 hours, so that the cells may adhere to each other. The culture medium is replaced with fresh medium after 24 hours. A series of twelve dilutions (10, 25, 50, 100, 150, 200, 250 300,350, 400,450 and 500 µg/ml) of each sample (extract and its fractions) were prepared. Each sample was exposed to cells at 37°C for 72 hours. After that the cell lines were exposed to different concentrations of plant extract and its various fractions for 72 hours under the same atmospheric conditions in the CO_2 incubator. After this incubation period, the culture media is again replaced with the fresh medium. After that 10 µl MTT solution (5mg/ml) made in phosphate buffer solution (PBS) was poured in to each well and the plate is again subjected to incubation at for 4 hours at 37°C in a CO_2 incubator. The DMSO was added to each well to dissolve any formazin crystals [15]. The optical density or absorbance of plant extract and its fractions was measured at 570 nm through micro-plate well spectrophotometer. Tamoxifen was used as positive control anduntreated cells were used as negative control. The results were recorded in triplicates to calculate the standard error means. The cytotoxicity of the samples was assessed through percentage growth inhibition, cell lines treated with Tamoxefen (standard), plant extract and its fractions against the untreated cell (control) by the formula given below:

$$\% \text{ Growth inhibition} = \frac{\text{Control- Sample Absorbance}}{\text{Control}} \times 100$$

Determination of IC50

IC50 values were determined through the cytotoxic activity results. From the results revealing the concentration at which 50% growth inhibition observed was considered as IC50[16]. The following criteria was used for categorizing the cytotoxic effect of plant extract and its fraction against the cancer cell lines under study on the basis of IC50 [17]:

IC50 ≤ 20 µg/ml = highly active, IC50 = 21 - 200 µg/ml = moderately active, IC50 = 201 - 500 µg/ml = weakly active, IC50 > 501 µg/ml = inactive

Statistical analysis

All the experimental results were presented as standard error of mean (± SEM) and analyzed by using statistical package for social sciences (SPSS) version.19, IBM Inc (USA).

Results

Phytochemical Screening

The phytochemical screening was carried out by procedures as mentioned in material and methods. This test confirmed the presence of flavonoids, steroids, saponin, triterpenoids and glycosides while alkaloids found absent (Table 1).

Table 1 Preliminary Test for Identification

Flavonoid Test	Alkaloid Test	Steroid Test	Saponin Test	Triterpenoids Test	Glycosides Test
+ve	-ve	+ve	+ve	+ve	+ve

Antibacterial Activity

The ethanolic crude extract and its fractions of *Wulfenia amherstiana* in different concentrations were tested for their antibacterial activities against both the gram positive. The negative control used was 0.5% DMSO, showed no activity against any bacteria. The results of antibacterial assay are shown in Table 2. From the results, it is clearly evident that the plant ethanolic extract demonstrated activity against *S.pneumoniae* with 41% inhibition at 600µg/ml. At the same concentration, it was also found effective against *B.subtilis* with a zone of inhibition of 8 ± 0.37 mm. The inhibitory zone of 10 ± 0.46 mm was observed against *S.aureus* at a concentration of 500µg/ml. The zone of inhibitions for *S.Flexneri, S. typhi* and *P.aeruginosa* were 8 ± 0.37, 11 ± 0.74 and 12mm respectively at 600µg/ml. The % inhibition calculation evidently revealed that the lowest crude extract activity was against *B.subtilis* (33%). The highest % inhibition (48%) was observed for *P.aeruginosa* and then comes the *S.aureus* (47%). The results (Table 2) clearly revealed that n-hexane was found most effective against *S. aureus* with a inhibitory zone 18±

0.49 mm which is near about that noted in case of cefazolin (21± 0.53mm) at 300µg/ml. While against *P.aeruginosa*, zone of inhibition observed was 18± 0.26 mm at the same concentration, which was somewhat close to that observed in case of Kanamycin (23± 0.74 mm).The n-hexane was found less effective against *B.subtilis* as % Inhibition was 56.5% at 300µg/ml. The % inhibition indicated the comparision of n-hexane activities against the various bacteria under study. Against *S.pneumoniae* , *S.typhi* and *S.flexneri*, the inhibitory zones were observed at 400µg/ml. The % inhibition against *Escherichia coli, S.typhi* and *B.subtilis* 72.7%, 62.5% and 65% respectively.The chloroform fraction showed maximum activity with 96% inhibition against the gram-ve *P.aeruginosa*. The inhibitory zone was appeared at 200µg/ml concentration. Whereas, it showed minimum activity (300µg/ml) against *Bacillus subtilis* as the % inhibition in this case was 75% which is lower in comparison to other bacterial strains. This fraction also showed impressive activity against *S.aureus*. Against *S.aureus* it showed 95.2% inhibition at 200µg/ml concentration. The chloroform fraction had inhibited the growth of *S.pneumoniae* and *S.flexneri* by 91% and 83.3%. This fraction indicated inhibition at 200µg/ml for *S.pneumoniae* and 300µg/ml against *S.typhi* and *S.flexneri*. The highest activity observed (Table 2) by n-Butanol fraction was against *S.aureus* with a zone of inhibition of 13± 0.61mm in contrast to 21± 0.53mm by kenamycin. This fraction caused 62% inhibition of *S.aureus* growth at 400µg/ml. Then,comes the *S.flexneri* where % inhibition was 60%. The inhibitory zone was 12±0.22 mm under the same concentration i.e. 400µg/ml.Whereas, the activities against *S.pneumoniae*, *S.typhi* and *P.aeruginosa* were found similar as the % inhibition was 50%. The concentration at which zones of inhibition were observed was 500µg/ml.The maximum activity shown by Ethyl acetate fraction was against *S.aureus* as indicated by % inhibition (71%) that was greater than the n-butanol fraction at 500µg/ml. While against the *P.aeruginosa* and *S.flexnari* inhibitory zones were 13±0.40 mm and 11±0.31 mm at 500µg/ml in comparison to inhibitory zones of 23±0.74 mm and 20±0.24 mm by Kenamycin. It was least effective against *S.typhi* with 50% inhibition at 600µg/ml concentration. However, the % inhibition against *S.pneumoniae* and *B.subtilis* 54% and 52% respectively at 600µg/ml concentration.

Antifungal Activity

The antifungal activity of *Wulfenia Artemisia* ethanolic crude extract and its fractions were tested against various fungal strains. The results are shown in Table 3. The drugs used as control were Miconazole and Amphotericin-A. While 0.5% DMSO was used as negative control and DMSO found ineffective against all fungal strains. The results (Table 3) revealed that ethanolic extract was most effective against *A.flavus* with the 55% inhibition (11± 0.10mm) at 500µg/ml and for *C.albicans* demonstrating 54.5% inhibition against it at 600 µg/ml with the zone of inhibition 12± 0.39 mm. The ethanolic extract found inactive against *F. solani*. It also showed low activity against *T.longifusis* and *M.canis* as indicated by 38% and 41% inhibition (zones of inhibition 10±0.25mm & 9±0.61mm) at 500µg/ml. While the zone of inhibition against

C.glabrata was 8± 0.43 at 600µg/ml at in comparision to 18±0.34 mm inhibitory zone by miconazole. The highest activity of n-hexane fraction was observed against *A.flavus* as the % Inhibition was 80% at 300µg/ml with the zone of inhibition 16± 0.40 mm as shown in Table 3. The inhibitory zone against *C.glabrata* was 14±0.32 mm in comparision to 18 ± 0.34mm of miconazole with

Table 2 Mean zone of inhibition (mm) against different bacterial strains by *Wulfenia amherstiana's* ethanolic extract and its various fractions

Bacterial Strains	Standard (mm)	Extract (mm)	% Inhibition	n-hexane (mm)	% Inhibition	Chloroform (mm)	% Inhibition	n-Butanol (mm)	% Inhibition	Ethyl acetate (mm)	% Inhibition
S.pneumoniae	22*± 0.35	9 ± 0.57	41	16±0.39	72.7	20± 0.30	91	11 ± 0.57	50	13± 0.46	54
B.subtilis NCTC 8236	24*± 0.61	8 ± 0.37	33	13.5± 0.35	56.25	18± 0.20	75	11 ± 0.13	46	12.5±0.17	50
S.aureus NCTC 6571	21*± 0.53	10± 0.46	47	18± 0.49	85.7	20± 0.48	95.20	13± 0.61	62	15± 0.24	71
S.Flexneri ATCC 12022	20**± 0.24	8 ± 0.37	40	13± 0.53	65	16± 0.65	80	12 ± 0.22	60	11± 0.31	55
P.aeruginosa	23**± 0.74	11±0.74	48	18± 0.26	78.2	22± 0.53	96	11.5± 0.46	50	13± 0.40	56
S.typhi ATCC 6539	24**± 0.61	11±0.59	46	15± 0.35	62.5	20± 0.60	83.30	12 ± 0.70	50	12± 0.59	50

Cefazolin*, Kenamycin**

The experimental values are means of triplicate, n = 3 with ± standard error mean (± SEM) the % inhibition of 77.7% at 300µg/ml which was greater than that observed for *C.albicans* with the % inhibition 72.7% at the same concentration. The lowest activity was observed against *F.solani* (10±0.53 mm) as % inhibition which was 55.5% at the concentration of 400µg/ml. Against *T. longifusis* the % inhibition was 69% at 400µg/ml and zone of inhibition observed was 18± 0.43 mm. The chloroform found more effective than the n-hexane fraction against all the fungal strains (Table 3). As it is evident from the results that it showed maximum activity against *A.flavus* (200µg/ml), *C.glabrata* (300µg/ml) and *C.albicans* (300µg/ml) where the % inhibition observed were 90%, 83.3% and 82% respectively. It had also shown activity against *F. solani*

with a zone of inhibition of 12±0.81 mm at 400µg/ml in comparision to 18±0.73 mm inhibitory zone by standard drug miconazole. The % inhibition was 66.6% in this case proving it less active in contrast to all the fungal strains. The n-butanol fraction also demonstrated antifungal activity but it was found less effective than the last two fractions. Among the fungal strains, *A.flavus* has shown maximum sensitivity against this fraction (70% inhibition). The zone of inhibition was 14± 0.47 mm at 500µg/ml. The lowest activity was demonstrated against *Fusarium solani* as the % inhibition was 44.4% and the inhibitory zone of 8± 0.55 mm was observed at 600µg/ml. While against *T. longifusis*, *M.canis* and *C.glabrata* the efficacy is similar as the % inhibition was 50% against all these strains. The concentrations at which zones of inhibition (13± 0.71 & 11± 0.31 mm) recorded were 500µg/ml for *T.longifusis* and *M.canis*. The antifungal potential of ethyl acetate was also tested against all the fungal strains under study (Table 3).It was least sensitive against *F.solani* and *M.canis* as shown by 44 & 45% inhibition in at 600 µg/ml. The efficacy against *A.flavus* and *C.glabrata* was greater than the rest of fungal strains. The % inhibition was more than 60% with inhibitory zones of 13± 0.64 mm (*A.flavus*) and 11± 0.41 mm(*C.glabrata*).

Cytotoxicity Assay by MTT method

The cytotoxicity activity of *Wulfenia amherstiana* ethanolic extract and its various fractions (n-hexane, chloroform, n-butanol and ethyl acetate) against human breast adenocarcinoma (MCF-7), human hepatocellular liver carcinoma (HepG-2) was evaluated through MTT assay. It was clearly evident from the results (Table 4) that the % growth inhibition was found to increase in a dose dependent manner. All the samples including the extract and fractions were capable of inhibiting the proliferation of cancer cells. But the samples were found more effective against MCF-7 cells than the Hepg-2 cells. The plant extract had showed most effective % inhibition in comparision to its fractions as the IC50 against MCF-7 cells was 50 µg/ml (% Growth inhibition
= 50.26 ± 2.7) and for HepG-2 the IC50 observed was 100 µg/ml with growth inhibition
50.59 ± 1.10%. Among the fractions chloroform and ethyl acetate were found more cytotoxic than the n-hexane and n-butanol. As the IC50 of chloroform against MCF-7 cancer cells was 100 µg/ml which was more than that of ethyl acetate (IC50 = 150 µg/ml). Same situation was observed against the HepG-2 cells as Chloroform's IC50 was 150 µg/ml and IC50 of ethyl acetate was 200 µg/ml. The IC50's of n-hexane and n-butanol against the MCF-7 cells were observed at 300 and 350 µg/ml respectively. While against the HepG-2 cancer cells 50% growth inhibition demonstrated by n-hexane at 350 µg/ml, while n-butanol showed it at 400 µg/ml results in Table 4.

Table 3 Mean zone of inhibition (mm) against different fungal strains by *Wulfenia amherstiana's* ethanolic extract and its various fractions

Bacterial Strains	Standard(mm)	Extract (mm)	% Inhibition	n-hexane (mm)	% Inhibition	Chloroform (mm)	% Inhibition	n-Butanol (mm)	% Inhibition	Ethyl acetate (mm)	% Inhibition
T.longifusis	26*± 0.61	10± 0.25	38	18± 0.43	69.00	20± 0.29	77%	13± 0.71	50%	11± 0.72	42%
A.flavus ATCC 32611	20**± 0.54	11± 0.10	55	16± 0.40	80.00	18± 0.34	90%	14± 0.47	70%	13± 0.64	65%
M.canis ATCC 11622	22*± 0.41	9± 0.61	41	14± 0.70	63.60	16± 0.51	72.70%	11± 0.31	50%	10± 0.31	45%
F.solani ATCC 11712	18*± 0.73	0± 0.00	0	10± 0.53	55.50	12± 0.81	66.60%	8± 0.55	44.40 %	8± 0.54	44%
C.albicans ATCC 2091	22*± 0.29	12± 0.39	54.5	16± 0.21	72.70	18± 0.34	82%	14± 0.31	63.60 %	13± 0.22	59%
C.glabrata ATCC60406	18*± 0.34	8± 0.43	44	14± 0.32	77.70	15± 0.46	83.30%	9± 0.62	50%	11± 0.41	61%

* Miconazole, ** Amphotericin-A
The experimental values are means of triplicate, n = 3 with ± standard error mean (± SEM)

Table 4. Cytotoxic Activity of Plant extract and its various fractions in terms of % growth Inhibition against the cell lines

Concentration µg/ml	Extract		n-hexane		Chloroform		n-Butanol		Ethyl Acetate		Tamoxifen	
	MCF-7	HepG-2	MCF-7	HepG-2	MCF-7	HepG-2	MCF-7	HepG-2	MCF-7	HepG-2	MCF-7	HepG-2
5	28± 1.60	25.32 ±0.52	18.2 ±0.82	15.10 ±0.90	26.21± 0.95	22.02 ±0.52	14.10 ±0.70	12.20 ±0.85	24.5±0.72	20.15 ±0.46	51.78 ±0.83	46.05± 0.93
10	37.45± 2.1	30.96 ±0.90	22.60 ±1.90	18.85 ±1.90	34.45± 1.5	27.06 ±0.86	18.05 ±0.90	14.80 ±0.99	32.05± 0.95	25.00 ±0.92	57.67 ±1.01	51.26± 0.73
25	43.20± 1.4	37.6 ±1.31	25.96 ±0.81	23.10 ±1.51	41.02± 0.85	34.10 ±1.02	22.82 ±0.88	19.20 ±0.80	39.8±0.6	32.40 ±1.20	64.81±1.3	58.45± 1.

									9		0	50
50	50.26±2.7	44.00 1.80	30.25 1.05	27.23 0.85	46.13±1.10	40.20 0.80	25.50 0.55	22.30 1.10	44.48±1.08	38.25 0.96	71.54 ±0.68	64.05±0.78
100	56.32±3.1	50.59 ±1.10	35.59 ±1.20	31.29 ±1.51	52.31±2.1	46.50 ±1.10	30.45 ±0.95	27.20 ±1.21	48.5±2.00	44.20 ±1.00	75.83 ±0.78	67.42±0.92
150	64.7±1.3	55.55 ±1.70	39.51 ±1.80	37.51 ±0.86	60.62±0.73	51.50 ±0.70	35.10 ±1.25	33.51 ±0.90	54.5±1.50	49.00 ±0.80	79.22 ±0.92	69.57±1.05
200	68.32±2.3	58.79 ±1.69	43.89 ±0.69	41.90 2.00	65.30±1.30	56.09 ±1.26	39.54 ±1.60	36.50 1.42	63.49±0.97	53.05 ±0.95	82.87 ±0.65	72.25±0.58
250	74.36±1.8	64.28 1.50	48.30 2.10	45.30 1.05	71.26±0.78	62.08 1.50	44.50 1.20	41.50 0.94	60.05±0.86	58.08 0.90	85.02 ±1.20	75.00±0.83
300	78.21±3.0	69.20 1.50	53.10 0.80	48.20 0.92	75.10±2.00	66.20 0.80	48.10 0.94	44.10 0.89	72.43±1.50	63.70 0.86	88.82 ±1.42	79.65±1.10
350	84.5±1.3	73.21 0.78	59.24 0.92	52.32 1.15	81.5±0.89	70.01 1.10	53.40 1.20	49.02 1.00	78.8±1.60	67.5 1.50	91.67 0.81	83.78±0.90
400	86.7 2.6	78.34 ± 1.03	64.34 ± 1.27	57.30 ± 2.05	84.7 1.30	75.60 ± 0.93	60.00 ± 1.27	52.80 ± 1.90	82.5±1.00	72.54 ± 0.89	95.95± 0.59	87.24±0.70
450	90 ± 2.0	84.21 1.20	72.21 1.20	65.91 1.52	88.50 1.00	81.71 0.80	69.80 ± 0.94	62.32 2.00	86.7±1.34	78.71 0.87	97.75± 0.99	92.5±1.01

DISCUSSION

The phytochemical screening clearly revealed that the ethanolic extract of *Wulfenia amherstiana* is composed of biologically active compounds flavonoids, steroids, saponins, glycosides and triterpenoids. The flavonoids are reported have antioxidant and antimicrobial activities[18] . In a study, a hydroalcoholic extract of *Carpolobia lutea* (Polygalaceae) was evaluated for antinociceptive anti-inflammatory activities. It was revealed that these activities were associated with the presence of tannins, terpenoids, saponins, cardiac glycosides, alkaloids and anthraquinones[19]. Similarly, the glycosides have been reported for their cytotoxic and anti- cancer activities in various studies[20, 21]. A plant known as *Platycodon grandiflorum* was found to exhibit cytotoxic activity against various human cancer cells including lung, ovary, CNS and colon cancer. This substantial anti-cancer activity was found associated with various triterpenoids saponins. All these compounds were (flavonoids, steroids, saponin, glycosides and triterpenoids) also found in the *Wulfenia amherstiana* which may be responsible for its various biological activities such as antimicrobial, antioxidant and anticancer activities. From the results of

antibacterial assays it was concluded that *Wulfenia amherstiana* possessed antibacterial activity. Its antimicrobial activities can be compared to other plants of same family such as *Antirrhinum majus*. Various extracts of this plant in different concentrations were used and their antibacterial, antifungal and cytotoxic activities were evaluated. The absolute methanol extract was found to inhibit the growth of *Pasturella multocida, E.coli, B.subtilis* and *S.aureus* significantly at higher concentrations. Its n-hexane fraction was found less effective against all these bacteria. The n-butanol fraction was inactive against *E.coli* even at higher concentration. Similarly, chloroform fraction of this plant was not able to effect the growth of *S. aureus, B. subtilis* [22] . Similarly, *Picrorhiza kurrooa*; another plant of this family was also studied for its antibacterial activities. The aqueous and methanol extracts of rhizomes of this plant have demonstrated activity against *S.aureus, B.subtilis, Micrococcus luteus,* and *E.coli* and *Pseudomonas aeruginosa*. Among these extracts only methanolic extract was found significantly efficacious only against *P. aeruginosa* and *S. aureus*[23]. In another study methanol, aqueous, n-hexane, acetone and ethanol extracts of *Picrorhiza kurrooa* rhizomes had shown potential activity against *B. cereus, E.coli*, *S.pneumoniae, Klebsiella pneumonia* (*K. pneumonia*), *S.aureus, Streptococcal pyogens* (*S.pyogens*) and *S.typhi)*. The aqueous extract demonstrated no activity. While acetone and hexane showed moderate activities against *B. cereus, E. coli, K. pneumonia, P. aeruginosa, S. aureus, S. pyogens* and *S. typhi*. The highest antibacterial activity was observed by methanolic extract against *S. aureus* and *P. aeruginosa*[24]. As the antifungal activity of *Wulfenia amherstiana* was also determined in our study. As there is no data available regarding this activity, but its activity can be compared to other plants of the same family.

In a study where aqueous and methanolic extracts of *Picrorhiza kurrooa* belonging to the same family were found active against all the fungal strains under study such as *C. albicans* and *A.niger*[25]. In another study methanolic extracts of various plants such as *Verbascum protractum* (*V. protractum*), *Verbascum bellum* (*V. bellum*), *Verbascum dalamanicum* (*V. dalamanicum*), *Scrophularia mersinensis* (*S.mersinensis*), *Scrophularia cryptophila* (*S. cryptophila*), *Pedicularis olympica* (*P. olympica*) *and Veronica lycica* (*V.lycica*) belonging to the same family *Scrophulariaceae* had shown antifungal activity against various standard fungal strains (yeast) including *Rhodotorula rubra* DSM 70403*, C.albicans* ATCC 10231 *and Kluyveromyces fragilis* ATCC 8608. It was concluded that *V. protractum, V. bellum* and *V. dalamanicum* were found effective against *Rhodotorula rubra, Candida albicans* and *Kluyveromyces fragilis*. Among these three members *V. bellum* was most active against the fungal strains under study[26]. On the basis of our results the plant *Wulfenia amherstiana* possessed impressive cytotoxic potential, as the plant's extract, its chloroform and ethyl acetate fractions have demonstrated anti-cancer activity against both human breast adenocarcinoma human and hepatocellular liver carcinoma cells. However, the anticancer activity against the human breast adenocarcinoma human cells was more than the hepatocellular liver carcinoma cells. Its cytotoxic activity can be compared to a plant known as *Picrorrhiza kurroa* belonging to the same family Scrophulariacea, It's methanolic extract had

shown activity against human breast cancer cell lines (MCF-7 cell lines) with IC50 = 61.86 in terms of % cell viability. Upon isolation of compounds from this plant extract revealed the presence of iridoid glycosides that were responsible for its anticancer activity [27]. The phytochemical screening of plant under study has also revealed the presence of glycosides that may be responsible for its cytotoxic activities. Another plant *Paulownia tomentosa* member of the same family[28] which has been found to contain various secondary metabolites principally flavonoids, glycosides, terpenoids, quinones and phenolic acids. The cytotoxic effect of plant extracts was found associated with prenylated and geranylated[29] flavonoids as they were the principal constituents. These flavonoids especially the prenylated ones have been evacuated for their activities against 20 cell lines[30].

Conclusion

The present study concluded that the *Wulfenia amherstiana* possesses potential antimicrobial activities. As its chloroform and ethylacetate fractions showed substantial antibacterial activity against gram positive bacteria *Staphylococcus aureus* and gram negative bacteria *Pseudomonas aeruginosa*. Both Chloroform and n-hexane fractions were found most efficacious against *Aspergillus flavus*. The ethanolic extract, its chloroform and ethyl acetate fractions found most cytotoxic against the cell lines under study providing evidence for the anticancer activity. The sum and substance of our study evidently demonstrated that the plant could provide novel anticancer compounds.

Conflicts of interest Statement

The authors declare no conflicts of interest

References

[1]. Cragg, G.M. and D.J. Newman, *A tale of two tumor targets: topoisomerase I and tubulin. The Wall and Wani contribution to cancer chemotherapy.* Journal of Natural Products, 2004. 67(2):p. 232-244.

[2]. Heinrich, M., et al., *Fundamentals of Pharmacognosy and Phytotherapy E-Book*2017: Elsevier Health Sciences.

[3]. Olmstead, R.G., *Whatever happened to the Scrophulariaceae.* Fremontia, 2002. 30(2): p. 13-22.

[4]. Riaz, M., M. Zia-Ul-Haq, and H.Z. Jaafar, *Common mullein, pharmacological and chemical aspects.* Revista brasileira de farmacognosia, 2013. 23(6): p. 948-959.

[5]. Lone, P., A. Bhardwaj, and F. Bahar, *Traditional knowledge on healing properties of plants in Bandipora district of Jammu and Kashmir, India.* Int. J. Recent Sci. Res, 2013. 4(11): p. 1755-1765.

[6]. Lone, P.A., A.K. Bhardwaj, and F.A. Bahar, *Study of indigenous/traditional medicinal plant knowledge-An endeavour towards new drug discovery.* African Journal of Traditional,

Complementary and Alternative Medicines, 2015. 12(2): p. 73-95.
[7]. Brahmachari, G., et al., *Evaluation of the antimicrobial potential of two flavonoids isolated from Limnophila plants.* Chemistry & biodiversity, 2011. 8(6): p. 1139-1151.
[8]. Riaz, M., et al., *In vitro antimicrobial, antioxidant, cytotoxicity and GC-MS analysis of Mazus goodenifolius.* Molecules, 2012. 17(12): p. 14275-14287.
[9]. Zulfiker, A.H., et al., *In vitro antibacterial, antifungal & cytotoxic activity of Scoparia dulcis L.*
Int J Pharm Pharm Sci, 2011. 3(2): p. 198-203.
[10]. Pooja, S. and G. Vidyasagar, *Phytochemical screening for secondary metabolites ofOpuntia dillenii Haw.* Journal of Medicinal Plants, 2016. 4(5): p. 39-43.
[11]. R., K. Nisha, and J. Kumar, *Preliminary phytochemical screening of methanolic extract of Clerodendron infortunatum.* IOSR J. Appl. Chem, 2014. 7(1): p. 10-3.
[12]. Balouiri, M., M. Sadiki, and S.K. Ibnsouda, *Methods for in vitro evaluating antimicrobial activity: A review.* Journal of Pharmaceutical Analysis, 2016. 6(2): p. 71-79.
[13]. Bhalodia, N.R. and V. Shukla, *Antibacterial and antifungal activities from leaf extracts of Cassia fistula l.: An ethnomedicinal plant.* Journal of advanced pharmaceutical technology & research, 2011. 2(2): p. 104.
[14]. Ogbole, O.O., P.A. Segun, and A.J. Adeniji, *In vitro cytotoxic activity of medicinal plants from Nigeria ethnomedicine on Rhabdomyosarcoma cancer cell line and HPLC analysis of active extracts.* BMC complementary and alternative medicine, 2017. 17(1): p. 494.
[15]. Bahuguna, A., et al., *MTT assay to evaluate the cytotoxic potential of a drug.* Bangladesh Journal of Pharmacology, 2017. 12(2): p. Online: Apr 8-2017.
[16]. Shahneh, F.Z., et al., *Inhibition of growth and induction of apoptosis in fibrosarcoma cell lines by Echinophora platyloba DC: in vitro analysis.* Advances in pharmacological sciences, 2013. 2013.
[17]. Srisawat, T., et al., *Phytochemical screening and cytotoxicity of crude extracts of Vatica diospyroides symington type LS.* Tropical Journal of Pharmaceutical Research, 2013. 12(1): p. 71-76.
[18]. Djouossi, M.G., et al., *Antimicrobial and antioxidant flavonoids from the leaves of Oncoba spinosa Forssk.(Salicaceae).* BMC complementary and alternative medicine, 2015. 15(1): p. 134.
[19]. Nwidu, L.L., B. Airhihen, and A. Ahmadu, *Anti-Inflammatory and Anti-Nociceptive Activities ofStem-Bark Extracts and Fractions of Carpolobia Lutea (Polygalaceae).* Journal of basic and clinical pharmacy, 2016. 8(1): p. 25.
[20]. Prassas, I. and E.P. Diamandis, *Novel therapeutic applications of cardiac glycosides.* Nature reviews Drug discovery, 2008. 7(11): p. 926.
[21]. Newman, R.A., et al., *Cardiac glycosides as novel cancer therapeutic agents.* Molecular interventions, 2008. 8(1): p. 36.
[22]. Riaz, M., et al., *Chemical Analysis, Cytotoxicity and Antimicrobial Studies by Snapdragon: A Medicinal Plant.* Asian Journal of Chemistry, 2013. 25(10): p. 5479.

[23]. Rathee, D., et al., *Phytochemical screening and antimicrobial activity of Picrorrhiza kurroa, an Indian traditional plant used to treat chronic diarrhea.* Arabian Journal of Chemistry, 2016. 9: p. S1307-S1313.
[24]. Kumar, P., et al., *In vitro antibacterial activities of Picrorhiza kurroa rhizome extract using agar well diffusion method.* Int J Curr Pharm Res, 2010. 2(1): p. 30-33.
[25]. Masood, M., et al., *Picrorhiza kurroa: An ethnopharmacologically important plant species of Himalayan region.* Pure and Applied Biology, 2015. 4(3): p. 407.
[26]. Dulger, B. and E. Ugurlu, *Evaluation of antimicrobial activity of some endemic Scrophulariaceae. members from Turkey.* Pharmaceutical Biology, 2005. 43(3): p. 275-279.
[27]. Rathee, D., et al., *Iridoid glycosides-Kutkin, Picroside I, and Kutkoside from Picrorrhiza kurroa Benth inhibits the invasion and migration of MCF-7 breast cancer cells through the down regulation of matrix metalloproteinases: 1st Cancer Update.* Arabian Journal of Chemistry, 2013. 6(1): p. 49-58.
[28]. Ali, S.A., et al., *The potential chemo preventive effect of ursolic acid isolated from Paulownia tomentosa, against N-diethylnitrosamine: initiated and promoted hepatocarcinogenesis.* Heliyon, 2019. 5(5): p. e01769.
[29]. Yuan, Z., et al., *Isolation and bioassay of herbicidal active ingredient from Paulownia tomentosa.* Chinese Journal of Pesticide Science, 2009. 2.
[30]. Schneiderová, K. and K. Šmejkal, *Phytochemical profile of Paulownia tomentosa (Thunb).Steud.* Phytochemistry reviews, 2015. 14(5): p. 799-833.